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Short communication

Expression and activity of recombinant proaerolysin derived from *Aeromonas hydrophila* cultured from diseased channel catfish

Dunhua Zhang *, Julia W. Pridgeon, Phillip H. Klesius

Aquatic Animal Health Research Unit, USDA-ARS, 990 Wire Road, Auburn, AL 36832, USA

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ABSTRACT

Aerolysin is one of the putative toxins in extracellular products (ECP) produced by *Aeromonas hydrophila*, an important pathogen of catfish. To better understand the molecular mechanism and mode of action of this toxin, proaerolysin-coding gene was cloned from the genomic DNA of an *A. hydrophila* strain, cultured from diseased channel catfish, and heterologously expressed in *E. coli*. Functional recombinant proaerolysin was obtained, revealing some unique properties. The purified recombinant proaerolysin was inactive but could be activated by treatment with furin, trypsin, and ECP although different treatments produced different cleavage profiles and resulted in differential hemolytic and cytotoxic activities. The highest activity was observed from aerolysin processed by furin while treatment of proaerolysin with trypsin and ECP resulted in reduced activities. The unprocessed proaerolysin, though not hemolytic *in vitro*, had the same cytopathic effect on cultured walking catfish gill cells as the furin-processed had. In *in vivo* assay, the recombinant proaerolysin was found to be lethal to catfish when injected *via* intraperitoneal (IP) route. The lethal toxicity was acute and dose-dependent, as observed in IP injection of live *A. hydrophila*. This is the first recombinant proaerolysin confirmed to be a virulence factor; the recombinant protein could be used to further evaluate virulence, pathogenicity and antigenicity associated with *A. hydrophila* infection.

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1. Introduction

Aerolysin was recognized as one of the virulence factors found in extracellular products (ECP) of *Aeromonas hydrophila*, an economically important pathogen of farmed fish (Roberts, 1993; Hemstreet, 2010). The aerolysin gene has hence been used as one of virulence markers to identify potentially pathogenic *Aeromonas* strains (Heuzenroeder et al., 1999; González-Serrano et al., 2004; Singh et al., 2008).

The role of aerolysin was considered to bind eukaryotic cell surface and form holes (channels), leading to destruction of membrane permeability and cell death (Howard and Buckley, 1982). The protein was, however, secreted as an inactive form, proaerolysin, which had to be activated by proteolytic cleavage (Howard and Buckley, 1985), releasing a C-terminal peptide and forming active oligomers (van der Goot et al., 1994). Trypsin (van der Goot et al., 1992), mammalian proprotein convertase (Abrami et al., 1998), and metalloprotease (Song et al., 2004) were shown to be able to process the proaerolysin.

The pathogenecity and immunogenicity of proaerolysin/aerolysin in catfish are not currently well understood mostly due to the difficulties in purifying large quantity of the bioactive protein from *A. hydrophila* culture. Over-expression and production of recombinant protein

* Corresponding author. Tel.: +1 334 887 3741; fax: +1 334 887 2983.
E-mail addresses: dunhua.zhang@ars.usda.gov, dhzhang8@gmail.com (D. Zhang).

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in *Escherichia coli* system should offer an achievable way to meet the need. Earlier, attempts to express pre-proaerolysin in *E. coli* have been made (Zhu et al., 2007; Singh et al., 2010) but true functions of the recombinant protein were not revealed.

In this study, a proaerolysin gene was cloned from a virulent strain of *A. hydrophila* and over-expressed in *E. coli*. Functional recombinant proaerolysin was, for the first time, obtained in ample quantity. Hemolytic and cytotoxic activities with or without proteolytic processing were able to be assessed; and the toxicity to catfish fingerlings was evaluated.

2. Materials and methods

2.1. Cloning of proaerolysin gene

Genomic DNA was isolated from a virulent strain of *A. hydrophila*, ML-10-51k, a kidney isolate from diseased channel catfish obtained during outbreak of motile aeromonad septicemia (MAS) in west Alabama (Hemstreet, 2010), using AquaPure genomic DNA isolation kit (BioRAD, Hercules, CA, USA). Two primers, targeting the encoding region of mature proaerolysin peptide, were synthesized according to the sequence of type strain ATCC 7966^T (GenBank# ABK36425; Seshadri et al., 2006). The forward primer, 5′-GCA GA*C CAT GG*C AGA GCC CGT CTA TCC AGA, was flanked with an *Nco* I restriction site at 5′ end (shown in italic) while the reversed one, 5′-CCT GG*C TCG AG*T TGA TTG GCA GCT *G*GC GTC A, was flanked with an *Xho* I site at 5′ end (shown in italic). The proaerolysin gene was PCR-amplifed using the above genomic DNA as template, digested with *Nco* I and *Xho* I, and cloned into pET28a vector plasmids (Novagen, Madison, WI, USA) at corresponding restriction sites. Recombinant pET28a plasmids were propagated in *E. coli* NovaBlue cells (Novagen) and purified for sequencing analysis. The cloned proaerolysin gene did not contain a stop codon at the 3′ end so that six consecutive histidine residues (His-tag) would fuse to the C-terminus of proaerolysin during expression.

2.2. Expression of proaerolysin gene in E. coli

Recombinant pET28a plasmids with correct insertion were used to transform competent cells of *E. coli* Rosetta 2(DE3) (Novagen). Over-expression of the target gene was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.4 mM, final concentration) to the bacterial culture with continuous shaking at 22 °C for 16–20 h. After induction, bacterial cells were harvested by centrifugation. Cell pellets were frozen at −80 °C until protein purification. Recombinant proaerolysin produced in *E. coli* was purified under native conditions using ProBond^{TM} Purification System (Invitrogen, Carlsbad, CA, USA). Buffer-exchange was performed after the protein was eluted from the binding resin using phosphate-buffer saline (PBS; Sigma–Aldrich, St. Louis, MO, USA) and 20 K MWCO-concentrator (Thermo Scientific, Rockford, IL, USA). The final protein concentration was estimated with the Bradford Reagent (Sigma–Aldrich) using bovine serum albumin (BSA) as the standard protein. The recombinant proaerolysin prepared in this study was referred to as rpArl or rArl (after proteolytic processing; see below) hereafter.

2.3. Protein electrophoresis and Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using NuPAGE 4–12% Bis-Tris pre-cast gel and MES/SDS running buffer (Invitrogen). Approximately 1.5–2.0 μg of protein were applied to each lane. After running, the gel was stained using SimplyBlue SafeStain (Invitrogen). For western blot, about 0.3 μg of protein were applied to each lane. After gel electrophoresis, proteins were electrically blotted to PVDF membrane and detected using Penta-His HRP conjugate and HRP staining reagents (Qiagen, MD, USA).

2.4. Processing of proaerolysin (rpArl)

Proteolytic processing of rpArl was performed in 100 μl of PBS (Sigma–Aldrich), containing 25 μg of rpArl and either 5 units of furin (New England Biolabs), 20 ng of trypsin (Sigma–Aldrich) or 0.2 μg of ECP of *A. hydrophila* liquid culture (The bacteria were shaking-cultured in tryptic soy broth at 28 °C until the OD_{600} reached to 2.2–2.5; after centrifugation, the supernatant was filtered through 0.2 μ membrane and concentrated using 10 K MWCO-concentrator (Thermo Scientific) and protein concentration was estimated with the Bradford Reagent). The amount of protease applied was pre-determined to be the minimum that could result in complete cleavage in short reaction time. Individual reactions were kept at room temperature (RT) for 24 h, during which aliquots of reactions were sampled and kept on ice or at 4 °C until use for analysis.

2.5. Activities of rpArl and rArl

An aliquot of 1.5 μl of individual reaction solutions, described above and incubated at RT for 15 min, was applied to the surface of 5% sheep blood agar (SBA, Remel, Lenexa, KS, USA), which was pre-spread with 10 μl of chloramphenicol (3.4 mg/ml) to prevent microbial growth. The agar plate was kept at RT for 1–5 days. The appearance of a yellowish-to-clear zone around the sample application site was the indication of hemolysis (halo formation).

The cytotoxic (cytolytic) activities of rpArl and rArl were evaluated by incubation of the protein with walking catfish gill cells (G1B). The G1B cells were purchased from the American Type Culture collection (ATCC, Manassas, VA, USA). The cells were cultured in F-12 medium (ATCC) containing 10% fetal bovine serum and 24 mM HEPES at 25 °C as described previously (Pridgeon et al., 2011). An aliquot of 150 μl gill cells (approximately 1.5×10^5 cells/ml) was dispensed to each well of a 96-well microtitier plate. The protease treated or untreated rpArl solution, described above and incubated at RT for 15 min, was diluted serially by twofold with PBS. An aliquot of 7.5 μl of each dilution was mixed into the gill cell suspension in triplicate. The microtiter plate was then incubated at 25 °C. Gill cell response was examined under an inverted microscope 3 h after exposure to the treatment, when

cells in controls settled to the bottom wall of the culture well and assumed morphological changes. Cytopathic effect or cytotoxic activity of the treatment was expressed as the cytotoxic titer (Howard and Buckley, 1985), the reciprocal of the highest dilution with which 95% or more cells lost the ability to attach the wall of the culture well and/or apparently lysed at 18 h after treatment.

Toxicity of rpArl to catfish fingerlings and LD_{50} (lethal dosage that causes 50% mortality) were determined by intraperitoneal (IP) injection using six dosages (0.25, 0.5, 1.0, 1.5, 3.0 and 6.0 μg per fish) in 100 μl sterile PBS. At each dose, there were ten fish (average weight = 5.6 ± 0.6 g). Heat-denatured rpArl (55 °C for 15 min, pre-determined inactivation conditions) at dosage of 6 μg per fish and PBS were served as controls. The assay was repeated three times. Averages of each determinant were plotted with Microsoft Excel to generate logarithmic trendline and LD_{50} was calculated from the trendline equation. Experimental catfish were maintained in aquaria tanks (57 l water) with 10 fish per tank. The source, rearing, and treatment of the experimental fish were described previously (Pridgeon et al., 2011).

3. Results

3.1. The structure of proaerolysin gene

A 1410-bp nucleotide sequence (KC263039) was cloned from the genomic DNA of *A. hydrophila* strain ML-10-51k. The proaerolysin coding sequence (*arl*) shared 96.4% identity with that of type strain ATCC 7966^T (Supplemental Figure 1). Among the variations, 45 of them were synonymous codon mutations and three resulted in amino acid changes. The putative proprotein convertase motif sequence was conserved. The *arl* gene had one open reading frame and could be conceptually translated into 470 amino acids with calculated molecular weight of 52,083.79 Da.

3.2. Production and processing of rpArl

Batch production of rpArl was achieved by IPTG induction and low temperature culture of transformed *E. coli* cells. The purified rpArl appeared to be homogenous (in control lanes of Fig. 1A) with apparent molecular weight of 53.1 kDa (including His-tag).

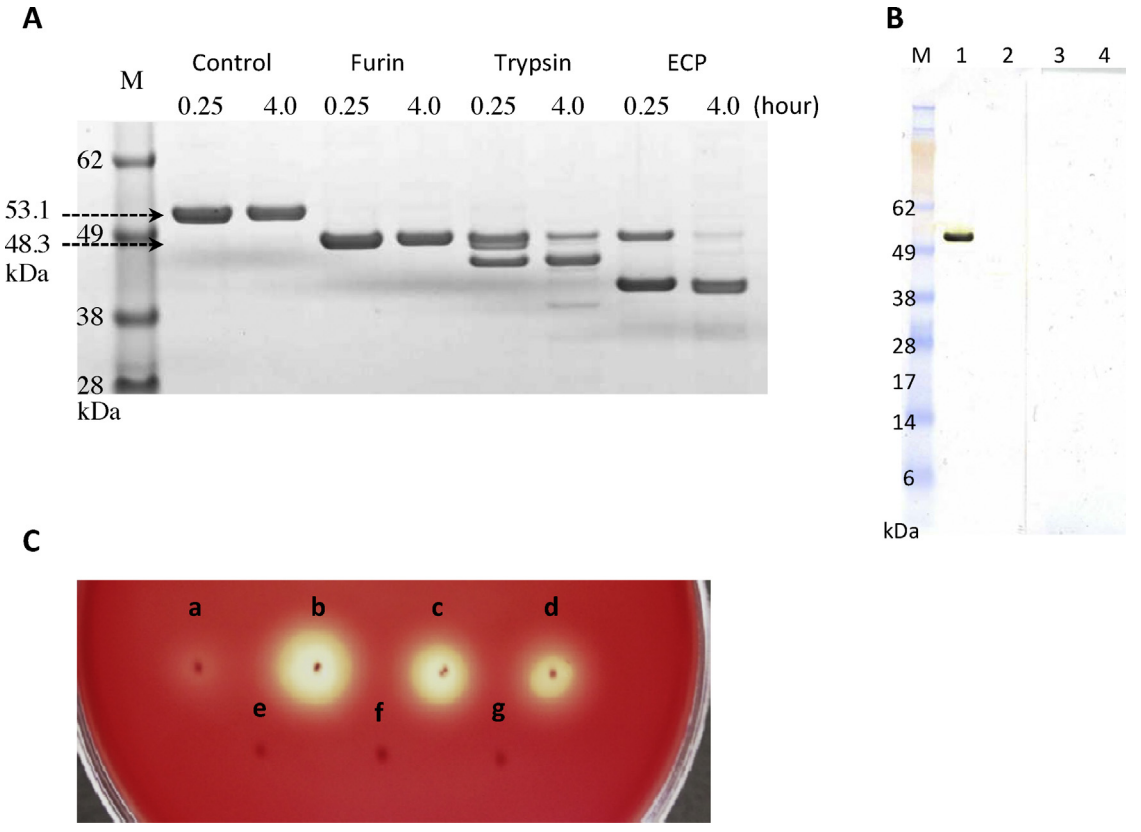

Fig. 1. SDS-PAGE (panel A), western analysis (panel B) and hemolytic activities (panel C) of recombinant proaerolysin (rpArl) and protease-treated products. (A) The rpArl (control) was incubated with furin, trypsin, or ECP of *A. hydrophila* liquid culture and samples were taken and analyzed at 15 min and 4 h after treatments. Calculated molecular weights of rpArl and rArl (post cleavage after KVRRTR^{432} by furin) were shown at the far left. (B) Samples analyzed in lanes 1–4 were the same as those treated with PBS (control), furin, trypsin, and ECPs for 4 hr at RT in panel A; proteins blotted to PVDF were incubated with anti-His HRP conjugate and detected using HRP staining reagents. M: SeeBlue Plus2 Prestained Standard (Invitrogen). (C) The samples applied in spots (a), (b), (c) and (d) are in correspondence with samples in control, furin, trypsin and ECP in 0.25 h lanes of panel (A), respectively. Samples applied in spots (e), (f) and (g) contained equivalent amount of furin, trypsin and ECP in (b), (c) and (d), respectively. The agar plate used was 5% sheep blood agar (Remel) and the image was taken 4 days after sample application.

The rpArl could be processed (cleaved) into lower molecular weight forms by either furin, trypsin, or ECPs of *A. hydrophila* liquid culture in as short as 15 min at RT (Fig. 1A). While there was no self-hydrolysis occurred in control (PBS only) even at prolonged incubation (>24 h at RT; data not shown), the treatment of furin resulted in complete cleavage of rpArl within 15 min, with one single lower molecular weight band shown in the gel (in furin lanes of Fig. 1A). The lost molecular weight roughly matched that of C-terminal peptide cleaved after KVRRTR^{432} (about 4.9 kDa, including His-tag). No additional cleavage was observed after 4 h (Fig. 1A) or 24 h (not shown) incubation. The cleavage of trypsin, however, produced at least three lower molecular weight bands within 15 min incubation (in trypsin lanes of Fig. 1A) with the highest one likely corresponding to the furin-produced band. Additional cleavage occurred as incubation time increased although the banding pattern at 4 h (Fig. 1A) remained unchanged after 24 h incubation (not shown). No cleavage by trypsin, however, was observed when the rpArl was still bond to the nickel-chelating resin during the course of the ProBond Purification described above (data not shown), indicating cleavage started from the C-terminal side of the protein as reported (Olsen et al., 2004). The ECP of the aerolysin-producing *A. hydrophila* caused different cleavage patterns from those of furin and trypsin (in ECP lanes of Fig. 1A). Following the appearance of a band equivalent of the size of furin cleavage, almost all were converted to a lower molecular weight band after 4 h-incubation and the 4-h-pattern remained unchanged at 24 h.

Western blot analysis showed that the anti-His HRP conjugate specifically bound the untreated rpArl (the control; lane 1 in Fig. 1B) but not those incubated for 4 h with either furin, trypsin, or ECP (lanes 2, 3 and 4, respectively, in Fig. 1B), indicating that peptide at C-terminus fused with His-tag was proteolytically removed and the corresponding band shown in Fig. 1A was the N-terminal part.

3.3. Hemolytic activity of rpArl and rArl

The hemolytic activities of rpArl (untreated) and rArl (treated with proteases) were evaluated on sheep blood agar. As shown in Fig. 1C (4 days after sample application), all protease treated samples, which were incubated at RT for 15 min, produced yellowish hemolytic halos (spots B to D) while the untreated rpArl had a light opaque halo (spot A). The intensity and size of furin-treated halo (spot B) were greater than those of trypsin (C) and ECP (D) treated haloes. The intensity and size of halo were apparently proportional to the amount of active protein applied as shown in Supplemental Figure 2.

3.4. Toxicity of rpArl and rArl

Both rpArl and rArl caused seriously cytopathic effects on cultured walking fish gill cells, from inability to attach the surface of the plate well to cell lysis (Supplemental Figure 3). The untreated proaerolysin (the same sample as that in 0.25-h control lane of Fig. 1A) was surprisingly as toxic as the furin-processed (0.25-h Furin lane of Fig. 1A); both had the highest cytopathic titers (Supplemental Table 1). The titer of trypsin-treated rpArl (0.25-h trypsin lane of Fig. 1A) was twofold lower while that of the ECP-treated (0.25-h ECPs lane of Fig. 1A) was fourfold lower.

Both rpArl and rArl were also found to be lethal to experimental catfish. Fish died between 3 and 4 h when lethal dose (2 μg per fish for either rpArl or rArl) was injected *via* IP route while no further mortality was observed in the following 4 weeks for those survived 4 h after exposure. Since the pre-data suggest that proaerolysin could be processed *in vivo* and was as toxic as the processed (data not shown), dose-dependent mortality rates were assessed using rpArl (Supplemental Figure 3). The LD_{50} for rpArl was about 1.2 μg per fish (or about 220 μg per kg by weight). No mortality was observed for controls (heat-denatured rpArl and PBS).

4. Discussion

The proaerolysin coding gene (*arl*) cloned from a field strain of *A. hydrophila* showed multiple codon mutations, including three amino acid changes, when aligned with the corresponding gene of the type strain ATCC 7966 (Supplemental Figure 1), indicating that isoforms of aerolysin exist in species *A. hydrophila*. Whether the sequence variation is related with the biological activity (such as virulence) is currently unknown but may be of importance since establishing an link between a virulence factor and pathogenesis of MAS associated with a field strain of *A. hydrophila* is still unequivocal (Khalil and Mansour, 1997; Santos et al., 1988; Singh et al., 2008). Further comparative analysis of different isoforms would be informative.

Under experiment conditions here described, the *E. coli* cells were capable of expressing the cloned *arl* gene and producing soluble proaerolysin with expected molecular weight. Purified proaerolysin was inactive and needed to be activated. The activation of proaerolysin by furin has been demonstrated (Abrami et al., 1998). In rpArl, there is a motif, K_{p6}-V_{p5}-R_{p4}-R_{p3}-T_{p2}-R_{p1}-$S_{p1'}$, in the C-terminal region which meets the minimal cleavage sequence, plus with a favorable lysine residue at P6 and a serine at the P1′ (Duckert et al., 2004). In congruence with the finding of Abrami et al. (1998), furin processing was shown to have one clear cut and generate the most active aerolysin with expected molecular weight (Fig. 1A). The cytotoxic concentration of activated aerolysin was estimated to be less than 5×10^{-10} M. Protease trypsin (at final concentration of 0.5 ng/μl) has also been shown to process and activate proaerolysin and no further breakdown products of mature aerolysin were seen even with the presence of a 10-fold excess of trypsin (Howard and Buckley, 1985). The results of this study, however, revealed that trypsin (at final concentration of 0.2 ng/μl) did activate proaerolysin but more than one product was observed on SDS-PAGE (trypsin lanes of Fig. 1A), and resulting products had lower activities than those activated by furin. Treatment of rpArl with ECP of *A. hydrophila* culture showed a different cleavage pattern from those of furin and trypsin (ECP lanes of Fig. 1A). It is well known that *A. hydrophila* secreted many proteases into culture media, some of which were

considered to be virulence factors (Sakai, 1985; Leung and Stevenson, 1988). An amino peptidase in *A. hydrophila* strain Ah65 (Howard and Buckley, 1985) and a metalloprotease in *A. veronii* biova sobria (Song et al., 2004) were reported to be able to process proaerolysin and generate a single mature aerolysin. However, the result of this study revealed that the secreted proteases of *A. hydrophila* may first cleave proaerolysin to a mature-like aerolysin and then generated a less-cleavable product (the ECP lanes of Fig. 1A). Since the activity of the ECP-cleaved products were fourfold lower than that of furin and twofold lower than that of trypsin, it suggests that the over-cleavage could be artificial and this phenomenon must be taken into account when working on the activities and profiles of *A. hydrophila* extracellular proteins. Unexpectedly, the unprocessed proaerolysin was as toxic as that processed by furin (Supplemental Table 1). A hypothesis was postulated to explain this type of activation mechanism in that some proprotein convertases may be tethered to the eukaryotic cell surface and proprotein processing could occur at the cell surface (Abrami et al., 1998).

Similar to *in vitro* cytotoxity assays, both proaerolysin (untreated) and aerolysin (protease-treated) were toxic to fish when injected *via* IP route, implying that there is an *in situ* activation mechanism for processing of the protoxin secreted by the bacterium. The rpArl/rArl-caused mortality was acute and dose-dependent, which resembles the mode of action observed in IP injection of live bacteria (Pridgeon and Klesius, 2011). It suggests that aerolysin could be the one or one of the virulence factors with which *A. hydrophila* kills the fish. The LD_{50} measured using rpArl was about 220 μg per kilogram of fish weight but, being the first report of an LD50 for a recombinant protoxin, the relative toxicity is not being able to compare at present. This, however, would serve as a reference for future related studies.

5. Conclusion

A functional proaerolysin was heterologously overproduced in *E. coli* and was proven to be a protoxin, which could be processed and activated *in vitro* and *in vivo*. The activated aerolysin was demonstrated to be highly hemolytic to sheep blood cells, cytotoxic to cultured walking fish gill cells and lethal to catfish. The recombinant proaerolysin would therefore be useful for further evaluating virulence, pathogenicity and antigenicity associated with *A. hydrophila* infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.04.023.

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